DETECTION AND CHARACTERIZATION OF PLASTIC DEGRADING MICROBES FROM RUMEN FLUID OF WATER BUFFALO, *Bubalus bubalis*

SYNOPSIS

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UZMA ADIL

Under the supervision of

PROF. ALKA PRAKASH

Department of Zoology, Faculty of Science

Dayalbagh Educational Institute (Deemed to be University)

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INTRODUCTION

Plastics are high molecular weight organic polymers which are obtained from different hydrocarbons and petroleum derivatives (Ahmed *et al*., 2018) and are highly durable due to their stable physical and chemical properties. Methods for their production are well established and they can be produced in large quantities at low cost. It is for this reason that they are extensively used worldwide (Barnes *et al*., 2009; Andrady, 2011; Hidalgo- Ruz *et al*., 2012). Polymers like polyethylene (PE), polyhydroxybutyrate (PHB), polyhydroxyalkanoate (PHA), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polylactic acid (PLA), and polybutylene succinate (PBS) have been produced for varied purposes because of their versatile nature (Andrady and Neal, 2009). The demand for plastics is on an exponential rise since the last few decades and every year approximately 367 million metric tons of plastic is produced as opposed to merely 1.5 million tons in 1950 (Tiseo, 2021). It has been recently reported that globally total plastic production has crossed 8 billion metric tons in 2018, and by 2050, it is expected to reach 34 billion metric tons (Lebreton and Andrady, 2019).

Plastic pollution, however, is majorly attributed to single use plastic products like bottles, plastic bags, packaging and cutlery items (Liu *et al*., 2021). Household/ industrial wastes, landfills, and human activities account for the major source of plastic bags in the environment. (Zhao *et al*., 2022). Only 9% of single use plastic is recycled, 12% is incinerated and consequently the remaining 79% piles up in the environment because it is considered to be unfit for recycling (Ahmed *et al*., 2018). This waste is dumped in the soil, with detrimental long term effects on soil fertility, marine ecosystem, and finally manifested in human health (Jambeck *et al*., 2015; Kumari *et al*., 2019).

While there is an urgent need to limit not only the production of plastics and their use, it is simultaneously important to find efficient means to manage plastics which are accumulating in every inch of space on earth annually at an alarming rate of approximately 8 million tonnes and is expected to grow fourfold by the middle of the 21st century (Laville and Taylor, 2017; Jambeck *et al.,* 2015; van Sebille *et al*., 2015; Gooljar, 2018).

Traditional physico-chemical methods of plastic disposal like incineration and landfilling are not environment friendly as they result in leaching of hazardous waste and toxic gases, thereby causing significant ecological issues. Additionally, these high molecular weight polymers are pre -treated with

harsh chemicals in the recycling process in order to deteriorate them, eventually making them more harmful for the environment (Hadad *et al*., 2005; Gautam *et al*., 2007; Okan *et al*., 2019). As opposed to traditional approaches, microorganisms have efficiently been able to degrade recalcitrant, mutagenic, or xenobiotic compounds such as polyaromatic hydrocarbons, pesticides, phenols, azo dyes, etc. (Christian *et al*., 2005; Meena *et al*., 2016; Narwal and Gupta, 2017). Additionally, several reports have emphasized on the ability of microbes to breakdown plastics in an economical and environment- friendly manner. Degradation by microbes depolymerizes these plastics into monomers and further mineralizes them into carbon dioxide, water and new biomass (Shah *et al*., 2008; Kreuger *et al*., 2015).

Various hydrolases with polyester- degrading abilities have been isolated from Actinobacteria e.g., *Thermobifida* spp. and from fungi including *Fusarium solani, Humicola insolens, Aspergillus* spp., and *Rhizopus* spp. (Ribitsch *et al*., 2013; Wei and Zimmermann, 2017). Polyethylene degrading bacteria like *Pseudomonas* sp., *Staphylococcus* sp., and *Bacillus*., sp has been isolated from soil samples (Singh *et al*., 2016). Microbes like *Paenibacillus* sp. and *Bacillus* sp. which are involved in degrading Polyethylene microplastics have been isolated from municipal landfill site. (Park and Kim 2019)

Bacteria like *Pseudomonas putida, Acinetobacter pittii,* and *Micrococcus luteus* have been able to perform biofragmentation of Low Density Polyethylene and use it as a source of carbon and energy. (Montazer *et al*., 2019). Fungi such as *Aspergillus, Zalerion, Fusarium* have also been able to degrade Polyethylene. (Pramila and Ramesh, 2011**;** Sheik *et al*., 2015**;** Paco *et al*., 2017)

Ideonella sakaienses is the most popular microbe involved in the degradation of Polyethylene Terephthalate (PET) (Yoshida *et al*., 2016). Actinobacteria like *Thermomonospora fusca* (Kleeberg *et al*., 1998), *Thermobifida alba* (Hu *et al*., 2010), *Sacharomonospora viridis* (Kawai *et al*., 2014)

The rumen microbiome comprises of bacteria, protozoa, archaea, phages and fungi, thereby making it the most diverse gut ecosystem in the animal kingdom (Huws *et al*., 2018; Newbold *et al*., 2020). Ruminants belong to the order *Artiodactyla*, and are cloven- hoofed mammals. They have 4 chambered stomach, among which the forestomach, called the reticulorumen is a large chamber having 50-100 litre capacity where ingested food is subjected to microbial degradation before the actual digestion (Gijzen *et* al., 1987). They rely on synergistic functioning of rumen microbes to degrade complex plant polysaccharides (Castillo- Gonzales *et al*., 2014). Extensive studies have been carried out to comprehend

enzymatic hydrolysis of polymers like hemicellulose, however enzymes with the ability to degrade other polymers is still under- explored. The ruminants have a dietary intake of natural plant polyesters like cutin, it is therefore probable that other enzymes possessing hydrolytic activities may also be found in the rumen (Mao *et al*., 2013).

Since rumen fluid includes not only one but multiple enzymes, along with a diverse microbiome, it's polyester degrading activity is high as opposed to published data for pure enzymes and/ or supernatants of single microorganisms. (Ribitsch *et al*., 2021)

India accounts for 43% of the world buffalo meat production, with Uttar Pradesh producing the most, followed by Andhra Pradesh and Maharashtra. There are 63 Indian Abattoirs cum Meat processing plants registered under APEDA (Agricultural and Processed Food Products Export Development Authority, Government of India) and 1072 regular slaughterhouses (APEDA 2022). Therefore, a large volume of rumen fluid is discarded as waste on a daily basis and can potentially cause eutrophication due to presence of ammonia and phosphates. (Sarteshnizi *et al*., 2018)

The proposed research aims to identify and isolate the specific microbe or a combination of microbes found in rumen fluid which can effectively degrade specific plastics, along with the by- products obtained and to prepare a microbial consortium that can synergistically degrade multiple plastics effectively within a short span of time.

REVIEW OF LITERATURE

Rumen microbes possess a high cellulolytic activity due to which they may have considerable potentials to bring about the anaerobic degradation of domestic refuse (Gijzen *et al*., 1987). Alterations in bacterial populations in the rumen of dairy cattle occur after adaptation to subacute ruminal acidosis (SARA) were determined with the help of 16S rRNA gene pyrosequencing (Mao *et al*., 2013**)**. The rumen has a complex ecosystem where diverse microorganisms such as bacteria, protozoa, and fungi work to digest the nutrients consumed by ruminants through fermentation process. There exists a symbiotic relationship among these groups of microorganisms due to their diverse nature, adaptability and interactions. The fermentation of dietary intake which comprises of lipids, carbohydrates and proteins is not 100% effective because of energy losses in the form of methane which is a greenhouse gas. In order to reduce methane production and improve the efficiency nutritional strategies like using additives in the diet such

as monensin, tallow, buffers, nitrogen compounds, probiotics in order to manipulate rumen fermentation (Gonzales *et al*., 2014). Studies by Huws *et al*., (2018) and Newbold *et al*., (2020) concluded that rumen possesses a great diversity of prokaryotic and eukaryotic microbes which include protozoa, anaerobic bacteria, fungi, methanogenic archaea, and phages. These microbes are involved in breaking down the ingested plant material and in turn provide energy to the host. Archaea, are involved in producing methane. Based on presence of natural polyesters present in the diet of ruminants, Ribitsch *et al*., in 2021**,** investigated synthetic polyester degrading enzymes from the rumen content of cattle. The group observed that rumen fluid, indeed was able to hydrolyse three synthetic polyesters, namely, poly (butylene adipate-co-terephthalate), poly (ethylene terephthalate) and bio-based polyester poly (ethylene furanoate) (PEF). The hydrolysis was analysed using SEM and HPLC.

Alongside these studies to bioremediate the various plastics, microbes were recognised as potential tools to degrade them. Kleeberg *et al*., in 1998 reported that aliphatic-aromatic co-polyesters synthesized from 1,4-butanediol, adipic acid, and terephthalic acid (BTA) can be degraded by mixed cultures. Actinomycetes strains identified as *Thermomonospora fusca* exhibited about 20-fold higher BTA degradation rates than usually observed in a common compost test. The underlying mechanism of white rot fungi in degrading structurally diverse xenobiotic organo-pollutants were extensively explained by Christian *et al*., 2005. Polyethylene was previously considered inert, but studies by Hadad *et al*., in 2005 concluded that it could be biodegraded by the action of the microbial strain *Brevibaccillus borstelensis*. The thermophilic bacterium was effectively isolated using enrichment culture methods and was found to be capable of utilizing polyethylene as the sole carbon and energy source. Further, Gautam *et al.,* in 2007 described the mechanism involved in the degradation of widely used synthetic polymeric foam along with the biodegradation pathways of a few synthetic polymers along with the physicochemical factors which impact the rate of biodegradation of synthetic foams. The existing methods of biodegradation of both synthetic as well as bio-based plastics along with the techniques used in the analysis of plastic degradation like carbon dioxide evolution, clear zone formation, weight loss measurement and the drawbacks associated with each of these techniques were reviewed by Shah *et al*., in 2008. Hu *et al*., 2010 isolated 100 bacterial strains from compost and categorized them into two groups, *Actinomycetes* (four genera) and *Bacillus* (three genera). Among these *Thermobifida*

alba possessed the ability to degrade aliphatic-aromatic copolyester film and decrease the polymer particle sizes when allowed to grow at 50°C on LB medium supplemented with polymer particles, yielding terephthalic acid.

Pramila and Ramesh in 2011 isolated *Acinetobacter baumannii* and determined its ability to degrade Low Density Polyethylene by performing Fourier Transform Infrared Spectroscopy (FTIR). Ribitsch *et al*., 2013 worked to fuse a cutinase from *Thermomyces cellulosilytica* with two different binding modules in order to enhance sorption which would further improve hydrolysis of the synthetic polymer polyethylene terephthalate (PET). The binding modules were also enzymes obtained from two different microbes. Sheik *et al*., 2015 isolated endophytic fungi from two endemic plants, *Psychotria flavida* and *Humboldtia brunonis* which produced laccase enzymes and inoculated them on the polythene and polypropylene films irradiated with different doses of radiation. Yoshida *et al*., in 2016 reported biodegradation of plastics by specialized bacteria *Ideonella sakaiensis*, which breaks down the plastic by using two enzymes to hydrolyze PET and a primary reaction intermediate, therby yielding basic building blocks for growth. Paco *et al*., 2017 tested the potential of the fungi *Zalerion maritimum* in the biodegradation of polyethylene and screened it by FTIR, Nuclear Magnetic Resonance (NMR) and Scanning Electron Microscope (SEM). Montazer *et al*., 2019 reported direct evidence for simultaneous bioconversion of LDPE plastic to biodegradable polyhydroxyalkanoate polymers by characterizing the ability of eight bacterial strains to utilize low-density polyethylene (LDPE) plastic in powdered form (untreated and without any additives) as a sole carbon source.

Andrady and Neal, 2009 described the history of plastics particularly polypropylene, polyethylene, polyvinyl chloride, polystyrene and polyethylene terephthalate, the rate of their production and consumption volumes. Versatility of these plastics with respect to their widespread use and concerns regarding their accumulation which further leads to littering of the environment has also been addressed. The problem of global plastic pollution and its rampant production was also addressed by Barnes *et al*., in 2009. The study also stated how plastic typically constitutes approximately 10% of discarded waste, yet they include a much greater proportion of the debris piling up on the coastline. The distribution of micro-plastic fragments has been on the rise since the last few decades. Andrady 2011 mentioned that the demand for plastics has been on a rise globally and stands at 245 million tonnes because of their

versatile nature, strength, light weight, and potentially transparent nature. They have variety of applications and are replacing conventionally used materials like glass, metal and paper due to their cost effective nature and equivalent or superior design. Hidalgo- Ruz 2012 reviewed numerous studies and compared methods being used for identifying as well as quantifying microplastics from the marine environment. Microplastics have been accumulating in the environment owing to their increasing use and popularity due to their physical and chemical properties. Jambeck *et al*., 2015; Andrady and Lebreton, 2019 linked studies on solid waste, economic status, and population density, and estimated the mass of land-based plastic waste which was entering the ocean. They calculated that 275 million metric tons (MT) of plastic waste was generated in 192 coastal countries in 2010, out of which 4.8 to 12.7 million MT was entering the ocean. The study has also proposed that if there is no improvement in the waste management infrastructure then the quantity of plastic waste entering the oceans would grow manifold. Kreuger *et al*., in 2015 stated that plastics have detrimental effects which threaten wildlife and exert effects on natural food webs, but signs for and knowledge on plastic biodegradation are limited. Microorganisms are the most promising candidates for an eventual bioremediation of environmental plastics. Previous studies have already reported various effects of microorganisms on many types of polymers, usually by enzymatic hydrolysis or oxidation. This study provided a comprehensive overview of the current knowledge on microbiological degradation of several of the most common plastic types., The study also illustrated the analytical challenges about the evaluation of plastic biodegradation and constraints in the effective biodegradation pathways. Van Sebille *et al*., 2015 reported that microplastic debris floating in the oceans has hazardous effects on the marine life. The study also estimated that in 2014 the accumulated number of microplastic particles range from 15 to 51 trillion particles, and weigh between 93 and 236 thousand metric tons, which accounts for only 1% of global plastic waste that was estimated to enter the ocean in the year 2010.

Narwal and Gupta, 2017 reported that accumulation of xenobiotic compounds comprising of pesticides, solvents, fuels, antibiotics, synthetic azodyes, pollutants (Dioxins and polychlorinated biphenyls), polyaromatic chlorinated and nitro- aromatic compounds. These compounds are mutagenic, teratogenic, carcinogenic and persist over a long time in the environment. The detoxification of these compounds is therefore important. Since microbes like bacteria and fungi have a wide range of catabolic degradation

pathways, these compounds can therefore serve as a sole energy and carbon source for these microbes. Wei and Zimmerman 2017, reported that natural materials have been replaced by petroleum based plastics due to their widespread uses across multiple aspects of life. However, due to their inertness, they have accumulated in the landfills and oceans, thereby becoming a global concern. Recently degradation or modification of these rather synthetic materials has been possible because of numerous microbial enzymes, therefore making them emerging candidates in order to develop biocatalytic recycling processes through which recovery of valuable raw materials can be made possible in a sustainable manner. Ahmed *et al*., 2018 classified plastics giving special attention to biodegradable plastics and their use. They also identified mechanisms involved in plastic degradation, insights into research on biodegradable plastics and microbes participating in biodegradation. Okan *et al*., 2019 highlighted sustainable approaches to reduce impacts of plastic waste on the environment.

Kumari *et al*., 2019 studied adherence of marine bacterial strain on Polyvinyl chloride, High Density Polyethylene, Low Density Polyethylene, which further led to their destabilization and degradation. Analytical and microscopic observations were used along with weight loss measurements. The interaction of bacteria with films led to changes in surface topography which were observed with the help of Atomic Force microscopy and Scanning Electron Microscopy.

Liu *et al*., 2021 stated that single use plastics constitute a large part of marine plastic litter, due to which they are now considered to be the world's most urgent environmental concern. Zhao *et al*., 2022 stated that households/ industrial wastes, landfills, and human activities are responsible for the major accumulation of plastic bags in the environment.

OBJECTIVES

1) Physical, chemical, biochemical and microbial assessment of rumen fluid.
2) Treatment of different plastic samples with microbes from rumen fluid.
3) Screening and identification of plastic degrading bacteria using conventional culture methods, followed by 16s ribosomal RNA sequencing.
4) Assessment of changes in surface topography after incubation with microbes using Scanning Electron Microscope (SEM), Atomic Force Microscopy (AFM) etc, and screening of the by-products obtained using biochemical techniques and chromatography.

5) Preparation of an optimum microbial consortium which can degrade some commonly used plastics.

PROPOSED METHODOLOGY

1. Sample collection: Rumen fluid of water buffalo from authorised slaughterhouse.
2. Physical, chemical, biochemical and microbial assessment of the rumen fluid:
    a) **Physical assessment**: Colour, odour, temperature.
    b) **Chemical assessment**: pH
    c) **Biochemical assessment:** molecules like proteins, lipids and carbohydrates.
    d) **Microbial assessment**: quantification of cells using Spectrophotometric analysis and nephelometric series.
3. Incubation of plastic samples in rumen fluid. (Quartinello *et al*., 2017)
4. Screening and isolation of microbes. (Das and Qin 2012)
5. Bacteriological characterization: Morphological and biochemical characterisation of bacteria which have been able to utilize different plastic samples as carbon source. (For identification of genera) (Poly *et al*., 2022)
6. Screening of plastic degrading bacteria using RAPD (Random Amplification of Polymorphic DNA) analysis followed by 16s ribosomal RNA sequencing followed by Basic Local Alignment Search Tool (BLAST). (McGovern E *et al.*, 2018)
7. Analysis of changes in surface topography indicating biodegradation of plastic films using Scanning Electron Microscope (SEM), Atomic Force Microscopy (AFM). (Sarmah and Rout, 2018; Quartinello *et al*., 2019; Park and Kim, 2019)
8. Screening of by- products obtained as a result of microbial action on plastics using FTIR spectroscopy, gas chromatography- mass spectrometry (GC- MS), HPLC- DAD (Quartinello *et* al., 2017; Sarmah and Rout, 2018; Muhonja *et al*., 2018; Park and Kim, 2019)
9. Based on results obtained, the most effective microbes in biodegradation will be utilised and their effect with respect to rate and extent of biodegradation will be determined.

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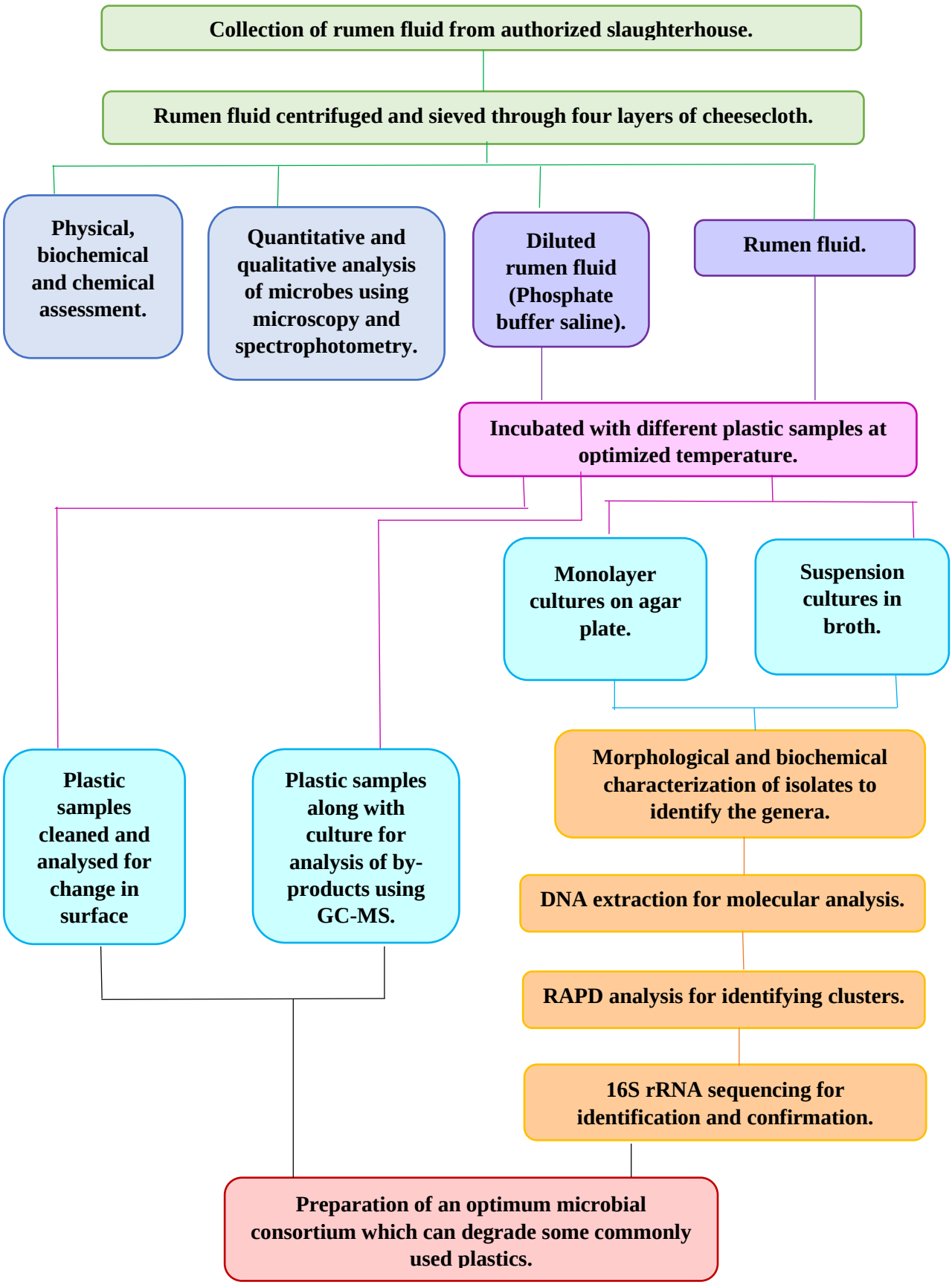
APPENDIX

PROPOSED WORK PLAN


Collection of rumen fluid from authorized slaughterhouse.
Rumen fluid centrifuged and sieved through four layers of cheesecloth.
Physical, biochemical and chemical assessment.
Quantitative and qualitative analysis of microbes using microscopy and spectrophotometry.
Diluted rumen fluid (Phosphate buffer saline).
Rumen fluid.
Incubated with different plastic samples at optimized temperature.
Monolayer cultures on agar plate.
Suspension cultures in broth.
Plastic samples cleaned and analysed for change in surface
Plastic samples along with culture for analysis of by-products using GC-MS.
Morphological and biochemical characterization of isolates to identify the genera.
DNA extraction for molecular analysis.
RAPD analysis for identifying clusters.
16S rRNA sequencing for identification and confirmation.
Preparation of an optimum microbial consortium which can degrade some commonly used plastics.